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# Construction, Expression, and Activities of L49-sFv-$\beta$-Lactamase, a Single-Chain Antibody Fusion Protein for Anticancer Prodrug Activation

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EXH. H

The L49 ($IgG_1$) monoclonal antibody binds to p97 (melanotransferrin), a tumor-selective antigen that is expressed on human melanomas and carcinomas. A recombinant fusion protein, L49-sFv-bL, that contains the antibody binding regions of L49 fused to the *Enterobacter cloacae* r2-1 $\beta$-lactamase (bL) was constructed, expressed, and purified to homogeneity in an *Escherichia coli* soluble expression system. The variable regions of L49 were cloned by reverse transcription–polymerase chain reaction from L49 hybridoma mRNA using signal sequence and constant region primers. Construction of the gene encoding L49-sFv-bL was accomplished by hybridization insertion of $V_H$, $V_L$, and sFv linker sequences onto a pET phagemid template containing the bL gene fused to the pelB leader sequence. Optimal soluble expression of L49-sFv-bL in *E. coli* was found to take place at 23 °C with 50 $\mu$M isopropyl $\beta$-D-thiogalactopyranoside induction and the use of the nonionic detergent Nonidet P-40 for isolation from the bacteria. Construction and expression of a soluble form of the p97 antigen in Chinese hamster ovary cells allowed affinity-based methods for analysis and purification of the fusion protein. Surface plasmon resonance, fluorescent activated cell sorting, and Michaelis–Menten kinetic analyses showed that L49-sFv-bL retained the antigen binding capability of monovalent L49 as well as the enzymatic activity of bL. *In vitro* experiments demonstrated that L49-sFv-bL bound to 3677 melanoma cells expressing the p97 antigen and effected the activation of 7-(4-carboxybutanamido)cephalosporin mustard (CCM), a cephalosporin nitrogen mustard prodrug. On the basis of these results, L49-sFv-bL was injected into nude mice with subcutaneous 3677 tumors, and localization was determined by measuring bL activity. Tumor to blood conjugate ratios of 13 and 150 were obtained 4 and 48 h post conjugate administration, respectively, and the tumor to liver, spleen, and kidney ratios were even higher. A chemically produced L49-Fab′-bL conjugate yielded a much lower tumor to blood ratio (5.6 at 72 h post administration) than L49-sFv-bL. Therapy experiments established that well-tolerated doses of L49-sFv-bL/CCM combinations resulted in cures of 3677 tumors in nude mice. The favorable pharmacokinetic properties of L49-sFv-bL allowed prodrug treatment to be initiated 12 h after the conjugate was administered. Thus, L49-sFv-bL appears to have promising characteristics for site-selective anticancer prodrug activation.

## INTRODUCTION

A considerable amount of attention has been directed toward the use of monoclonal antibody–enzyme conjugates in combination with suitable prodrugs for the selective delivery of chemotherapeutic agents to tumors (*1–3*). The monoclonal antibody (mAb[1]) portions of these immunoconjugates recognize tumor-selective antigens and are capable of delivering the enzymes to tumor cell surfaces. Once tumor localization and systemic conjugate clearance have taken place, a prodrug form of a chemotherapeutic agent is administered, which is converted into an active drug by the targeted enzyme. This leads to the selective delivery of anticancer drugs to sites of neoplasia. Pharmacokinetic studies have shown that the intratumoral drug concentrations resulting fro[m] enzyme/prodrug combinations can be significantl[y] than that achieved by systemic drug admin[istration] (*4–6*). This probably accounts for the observed a[ntitumor] activities, which include complete tumor regress[ions and] cures in a number of different models for huma[n cancer] (*7–10*).

Our recent research has focused on the use o[f antibod]ies against the human p97 (melanotransferri[n]) antigen for the delivery of $\beta$-lactamase (bL) to tu[mors] (*10*). This antigen has been found to be overe[xpressed] on a majority of clinical melanoma isolates a[nd] observed on human carcinomas (*11–14*). Si[gnificant] antitumor activities have been obtained using [a com]bination of a chemically conjugated anti-p97 Fa[b′-bL and] CCM (*10*), a cephalosporin containing prodru[g] (Figure 1). These effects were observed in a [...] tumor model that was resistant to the activitie[s ...]

A critical aspect of this targeting strategy [is] the ability of the mAb to selectively deliver th[e enzyme] to tumor cells and then clear from the system[ic circula]tion before the prodrug is administered. The [pharma]cokinetics of the immunoconjugate can be gre[atly influ]enced by the nature, valency, and molecular [size of] the mAb and also by the way that the enzyme i[s attached.] Typically, mAb–enzyme conjugates are prepa[red ...]

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[1] Abbreviations: bL, $\beta$-lactamase; CHO, Chinese hamster ovary; CCM, 7-(4-carboxybutanamido)cephalosporin mustard; FITC, fluorescein isothiocyanate; IMDM, Iscove's Modified Dulbecco's Medium; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; mAb, monoclonal antibody; PBS, 10 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4; PCR, polymerase chain reaction; PDM, phenylenediamine mustard; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; sp97, soluble p97 antigen; Tris, tris(hydroxymethyl)aminomethane.

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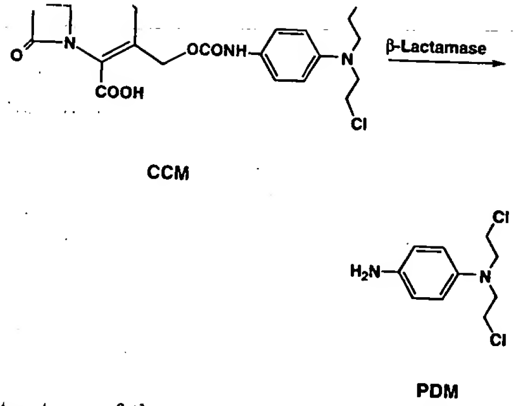


Figure 1. Structures of the cephalosporin mustard prodrug CCM and the parent drug phenylenediamine mustard PDM.

bifunctional cross-linking reagents that react with exposed amino acid residues on the individual proteins. Immunoconjugates produced in this manner are heterogeneous due to the inherent lack of regiospecificity of the cross-linking reagents. In addition, these conjugates are typically isolated in low yields. Although recent papers describe alternative coupling chemistries that can afford higher yields of more homogeneous immunoconjugates (*15–17*), these methods still involve chemical modification steps that contribute to product heterogeneity.

Recombinant technology offers an alternative method for producing homogeneous mAb−enzyme fusion proteins that can be designed to have appropriate pharmacokinetic properties for prodrug activation. There have recently been reports of the production, characterization, and activities of recombinant Fab, sFv, and disulfide-stabilized Fv−enzyme fusion proteins (*4, 18–20*). In this paper, we describe the construction, expression, and characteristics of L49-sFv-bL, an antibody β-lactamase fusion protein that binds to the p97 antigen. We also detail the construction and expression of sp97, a soluble form of the p97 antigen that has proven to be useful for conjugate analysis and purification. *In vitro* and *in vivo* experiments are presented that illustrate the ability of L49-sFv-bL to activate the phenylenediamine mustard prodrug CCM on p97 positive human melanoma cells, selectively localize in human tumor xenografts in nude mice, and induce regressions and cures of established tumors when combined with CCM.

## MATERIALS AND METHODS

**Materials.** The *Enterobacter cloacae* P99 bL gene was obtained from the plasmid pNU363 (*21*) and subjected to codon-based mutagenesis at the nucleotides corresponding to amino acids 286−290. The r2-1 bL mutant, which has the sequence TSFGN at positions 286−290, was selected from the resulting library and displayed greater enzymatic activity than the wild type enzyme using cephalosporin doxorubicin as the substrate (*22*). L49-Fab′-bL was prepared as previously described by combining thiol-containing Fab′ fragments of the antibodies with maleimide-substituted bL, forming a thioether link between the two proteins (*23*). CCM (*24*) and PDM (*25*) were prepared as previously described.

**Isolation and Characterization of the L49 mAb.** The L49-producing hybridoma was developed using standard techniques as previously described for the generation of other hybridomas (*26*). Balb/C mice were immunized repeatedly with the H2981 (lung carcinoma), [illegible] (lung carcinoma), and W56 (melanoma) cell lines, all of which were derived from human tumors. Spleen cells from immunized mice were fused with the cell line P3X63-Ag8.563 (*26*) that was transfected with the neomycin resistance gene. Standard selection and cloning yielded a hybridoma producing the L49 $IgG_1$ mAb.

Scatchard analysis of L49 binding was performed by radiolabeling the mAb with [$^{125}$I]Iodogen to a specific activity of 0.3 mCi/mg of protein. 3677 melanoma cells (*10*) in 96-well plates (13 000 cells/well) were incubated with 0.03−10 nM [$^{125}$I]L49 for 30 min on ice, and then the cells were separated from unbound radioactivity by centrifugation through silicon oil. The tubes were frozen, the cell pellet was cut from the supernatant, and both fractions were counted in a gamma counter. Binding affinity and the number of sites per cell were determined by Scatchard analysis (*27*).

**Soluble p97 (sp97).** A secreted form of p97 (sp97) was made utilizing PCR-based mutagenesis to introduce a stop codon after cysteine 709 (*14*), three amino acids upstream of the glycophosphatidylinositol anchor domain (*28, 29*). The 3′ oligonucleotide used in the PCR reaction contained the mutation changing the S710 codon to a stop codon. Coding sequences for 29 amino acids, including the membrane anchor region, were deleted from the carboxyl terminus of wild type p97. Cloning and expression of sp97 were accomplished using a glutamine synthetase gene as an amplifiable marker in CHO cells (*30*). The sp97 gene was cloned into pEE14 (*31*) and transfected into CHO-K1 cells by calcium phosphate coprecipitation. Transformants were initially selected for resistance to 25 μM methionine sulfoximine, and sp97-secreting colonies were selected for amplification at drug concentrations of 100, 250, and 500 μM. The selection and amplification medium used was Glasgow Minimum Essential Medium without L-glutamine, tryptose phosphate broth, or sodium bicarbonate supplemented with 10% dialyzed fetal bovine serum.

A cloned CHO cell line secreting sp97 was cultured in 10 shelf cell factories. Soluble p97 was isolated from the medium on a 96.5 immunoaffinity chromatography column as described previously for the purification of wild type p97 from melanoma cells (*32*). Small amounts of residual contaminants were removed by gel filtration on a Sephacryl S300 HR column (Pharmacia LKB) using PBS as eluant. Solutions containing sp97 were concentrated by ultrafiltration to 1−5 mg/mL, sterilized by passage through a 0.1 μm filter, and stored at 2−8 °C for up to 6 months without noticeable loss of biochemical or biological activity.

**Cloning of L49 Variable Regions and sFv Construction.** Construction of L49-sFv-bL by hybridization insertion was performed with materials and protocols from the Bio-Rad M13 mutagenesis kit, except for isolation of single-stranded phagemid template (Qiagen M13 kit, M13K07 helper phage). The variable regions of the L49 mAb were cloned from the corresponding hybridoma mRNA by RT-PCR (Perkin-Elmer GeneAmp reagents and Model 9600 thermal cycler), using random hexamer primed reverse transcription reactions and signal sequence and constant region PCR primers (*33*). Construction of L49-sFv-bL began with a single-stranded template of the pET-26b phagemid containing the r2-1 mutant of the *E. cloacae* P99 bL gene (*22*) fused to the pelB leader sequence. Hybridization mutagenesis was used to insert the 218 linker sequence (*34*) (chemically synthesized oligonucleotide, 5′-TTCTGACACTGGCGTGCCCTTGGTAGAGCCTTCGCCAGAGCCCGGTTTGCCAGAGCCGGACGTCGAGCCGGCCATCGCCGGCTG-3′) and full $V_H$ and $V_L$ region sequences (oligonucleotides produced by asymmetric PCR; $V_H$ forward primer, 5′-CCAGCCGGCGATGGCCGAGGTGCAGCTTCAGGAGT-

| sample | $k_{on}$ ($M^{-1}\cdot s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (nM) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M$) |
|---|---|---|---|---|---|
| L49 Fab' | $2.3 \times 10^5$ | $1.7 \times 10^{-4}$ | 0.73 | na[b] | na |
| r2-1 bL[c] | na | na | na | 261 | 19 |
| L49-Fab'-bL[d] | $1.8 \times 10^5$ | $2.4 \times 10^{-4}$ | 1.3 | nd[e] | nd |
| L49-sFv-bL[c] | $4.1 \times 10^5$ | $4.2 \times 10^{-4}$ | 1.0 | 232 | 19 |

[a] Values shown are the average of a minimum of two independent experiments, except for L49-Fab'-bL (binding experiment performed once). The range of values obtained in Michaelis–Menten kinetic analyses was within 5% of the means. Nitrocefin was used as an enzyme substrate. [b] Not applicable. [c] The r2-1 bL contains mutations at positions 286–290 compared to the wild type enzyme (*22*). [d] Chemically prepared conjugate containing the wild type enzyme. [e] Not determined.

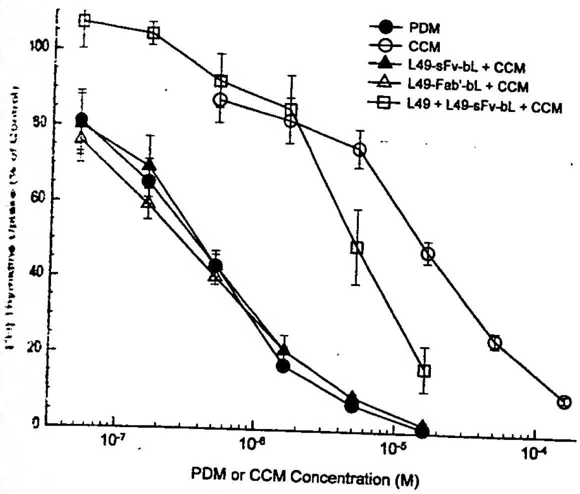


**Figure 7.** Cytotoxic effects of mAb–bL + CCM combinations on 3677 melanoma cells as determined by the incorporation of [³H]thymidine into DNA. 3677 cells were incubated with the mAb-bL conjugates, washed, and treated with CCM for 1 h. The effects were compared to cells treated with CCM or PDM for 1 h without prior conjugate exposure and to cells that were treated with saturating amounts of unconjugated L49 prior to conjugate treatment.

(Table 1). Michaelis–Menten kinetic analyses confirmed that the fusion protein retained the full enzymatic activity of the mutant bL enzyme from which it was derived (*22*). Thus, both the binding of the L49 mAb and the enzymatic activity of the *E. cloacae* r2-1 bL were preserved in the fusion protein.

The cytotoxic effects of L49-sFv-bL in combination with CCM were determined on 3677 human melanoma cells, which express the p97 antigen (Figure 7). The experiments were performed by treating the cells with the conjugates, washing off unbound material, adding various concentrations of CCM, and using [$^3$H]thymidine incorporation as a measure of cytotoxic activity. The prodrug CCM ($IC_{50} = 16\ \mu M$) was approximately 50-fold less toxic to 3677 cells than the drug PDM ($IC_{50} = 0.3\ \mu M$). As expected, L49-sFv-bL and L49-Fab'-bL were equally effective at prodrug activation, and the combinations were equivalent in activity to PDM. This indicates that prodrug conversion by both conjugates was efficient under the conditions tested. In addition, it was found that activation was immunologically specific, since L49-sFv-bL did not activate CCM on cells that were saturated with unconjugated L49 before being exposed to the fusion protein.

***In Vivo* Localization.** Biodistribution studies of L49-sFv-bL and L49-Fab'-bL were carried out in nude mice bearing sc 3677 melanoma tumor xenografts. The conjugates were injected iv, and at various time points

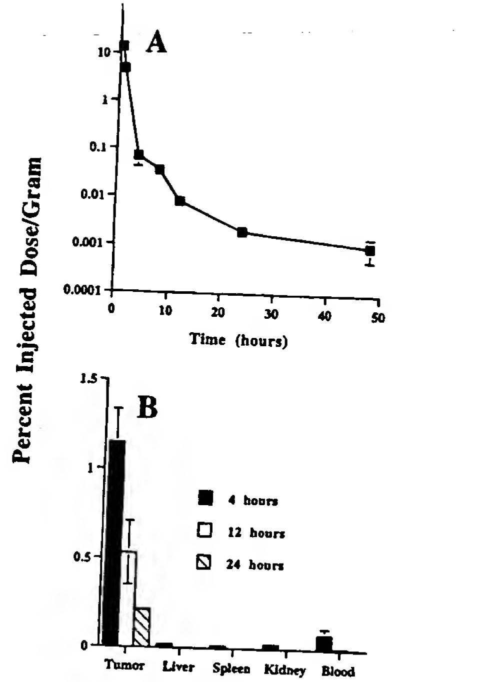


**Figure 8.** Pharmacokinetics of L49-sFv-bL in nude mice (three animals/group). L49-sFv-bL was injected intravenously, tissues were removed and extracted at the indicated times, and the $\beta$-lactamase activity was determined using nitrocefin as a substrate. (A) Clearance of L49-sFv-bL from the blood. Injected dose was 4 mg/kg. (B) L49-sFv-bL levels in subcutaneous 3677 melanoma tumors and in normal tissues. Injected dose was 1 mg/kg.

tissues were removed and extracted under alkaline conditions to disrupt antigen–antibody interactions. The samples were then trapped with polyclonal antiserum to bL, and bL activity was measured using nitrocefin as a colorimetric indicator (*37*). Control experiments in which L49-sFv-bL was directly injected into excised tumors and tissues indicated that this extraction procedure recovered 90% of the injected bL activity.

L49-sFv-bL cleared very rapidly from the blood (Figure 8A). The initial and terminal clearance half-lives ($t_{1/2}\alpha$ and $t_{1/2}\beta$, respectively) were 0.3 and 2.5 h, respectively, leading to a $10^4$ reduction of L49-sFv-bL blood levels within 24 h of conjugate administration. In spite of this rapid clearance, relatively high intratumoral levels of L49-sFv-bL were measured compared to normal tissues, and the ratio remained high for 24 h (Figure 8B). At 4 h post L49-sFv-bL administration, the tumor to blood ratio was 13:1. The ratio increased substantially with time and was 105:1 within 24 h of conjugate administration (Table 2). Similar results were obtained using L49-sFv-bL doses of 4 mg/kg. At this dose, very high tumor to blood ratios (141–150:1) were measured 24–48 h after the conjugate was administered. Interestingly, chemically produced L49-Fab'-bL cleared quite slowly from the blood and had only a 5.6:1 tumor to blood ratio 72 h after administration. Thus, L49-sFv-bL localizes in tumors, clears rapidly from the systemic circulation, and has significantly improved pharmacokinetic properties compared to the chemically produced L49-Fab'-bL conjugate.

**Therapeutic Activity.** *In vivo* therapy experiments were performed using the L49-sFv-bL/CCM combination in nude mice with established sc 3677 tumors. This particular tumor model has previously been shown to be resistant to treatment with doxorubicin, PDM, and CCM (*10*). In the experiments reported here, conjugate treatment was initiated 7–8 days after tumor implant, at which time the tumors were approximately 130 mm³ in

... 2. Tissue Distribution of Immunoconjugates

| treatment[a] | time (h) | % injected dose/g (SD) tumor | liver | spleen | kidney | blood |
|---|---|---|---|---|---|---|
| L49-sFv-bL, 1 mg/kg | 4 | 1.1 (0.2) | 0.021 (0.002) | 0.014 (0.008) | 0.027 (0.015) | 0.084 (0.04 |
| tumor/tissue ratios | | 1 | 52 | 79 | 41 | 13 |
| L49-sFv-bL, 1 mg/kg | 12 | 0.53 (0.17) | <0.003 | <0.003 | <0.003 | 0.008 (0.00 |
| tumor/tissue ratios | | 1 | >177 | >177 | >177 | 66 |
| L49-sFv-bL, 1 mg/kg | 24 | 0.21 (0.01) | <0.003 | <0.003 | <0.003 | 0.002 (0.00 |
| tumor/tissue ratios | | 1 | >70 | >70 | >70 | 105 |
| L49-sFv-bL, 4 mg/kg | 12 | 0.73 (0.02) | <0.003 | <0.003 | <0.003 | 0.009 (0.00 |
| tumor/tissue ratios | | 1 | >240 | >240 | >240 | 81 |
| L49-sFv-bL, 4 mg/kg | 24 | 0.29 (0.05) | <0.001 | <0.001 | <0.001 | 0.002 (0.00 |
| tumor/tissue ratios | | 1 | >290 | >290 | >290 | 141 |
| L49-sFv-bL, 4 mg/kg | 48 | 0.15 (0.07) | nd[b] | nd | nd | 0.001 (0.000 |
| tumor/tissue ratios | | 1 | | | | 150 |
| L49-Fab′-bL, 1.8 mg/kg | 72 | 0.28 (0.26) | 0.015 (0.003) | 0.010 (0.006) | 0.016 (0.005) | 0.05 (0.015) |
| tumor/tissue ratios | | 1 | 19 | 28 | 18 | 5.6 |

[a] Mice (three animals/group) were injected with conjugates, and at the times indicated, tissues were excised and extracted to remo the conjugate. The percent injected dose was based on the measured bL activity compared to standard curves obtained from extract tissues that were spiked with known amounts of L49-sFv-bL or L49-Fab′-bL. [b] Not determined.

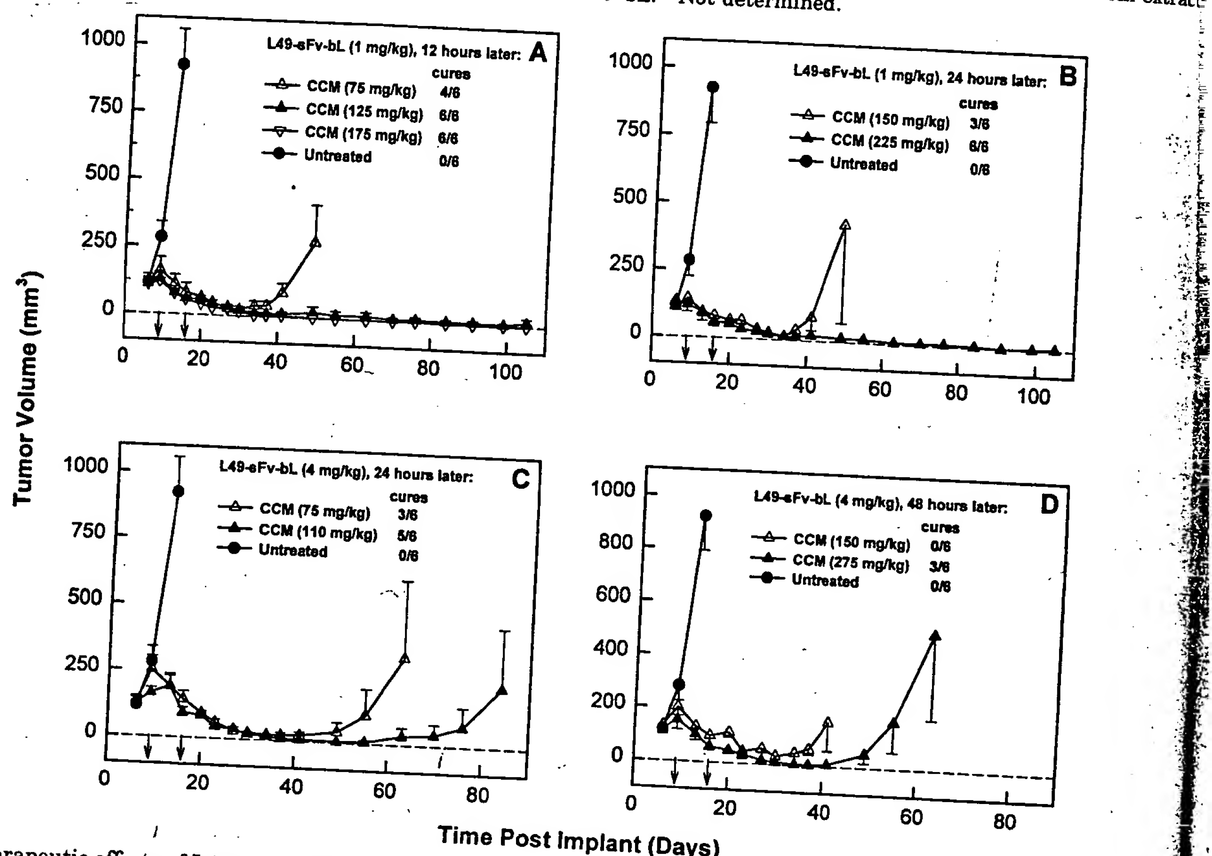


**Figure 9.** Therapeutic effects of L49-sFv-bL/CCM combinations in nude mice (six mice/group) with sc 3677 melanoma xenografts. Conjugates were injected, followed at various times by CCM (arrows on the *X*-axis). The average tumor volumes are reported until most or all of the animals were cured (tumors that became nonpalpable for ≥10 tumor volume doubling times) or until an animal was removed from the experiment due to tumor outgrowth. (A) L49-sFv-bL (1 mg/kg/injection) 12 h before CCM; (B) L49-sFv-bL (1 mg/kg/injection) 24 h before CCM; (C) L49-sFv-bL (4 mg/kg/injection) 24 h before CCM; (D) L49-sFv-bL (4 mg/kg/injection) 48 h before CCM.

volume. CCM was then administered 12, 24, or 48 h later, and the treatment protocol was repeated after 1 week. Maximum tolerated doses are defined as those that led to <20% weight loss and no treatment-related deaths and were within 50% of the dose at which such events took place. A tumor was considered as having been cured once it was not palpable for at least 10 tumor volume doubling times, based on the tumor growth of untreated animals (tumor volume doubling time was 4 days). If an animal was removed from the experiment because of tumor growth, the data from the entire group were no longer plotted, but the remaining animals were followed for tumor size and general health.

The maximum tolerated doses of CCM (300 mg/kg/injection) and PDM (3 mg/kg/injection) when administered weekly for three rounds induced 2 and 6 day delays in tumor outgrowth, respectively (data not shown). In contrast, pronounced antitumor activity was obtained in mice that received L49-sFv-bL prior to treatment with CCM (Figure 9). Therapeutic efficacy was schedule and dose dependent. Tumor cures were obtained in all of the animals that received CCM (125 and 175 mg/kg/injection) 12 h after treatment with L49-sFv-bL (Figure 9A). In this dosing schedule, significant antitumor activity including four cures in the group of six mice was obtained when the CCM dose was reduced to 75 mg/kg/injection.

remaining two animals in this group had tumors that ...ergwent partial regressions but eventually began to ... after the last prodrug treatment. There were no ...parent toxicities in any of these treatment groups.

...ignificant antitumor activity could also be achieved ...en the prodrug was administered 24 h post conjugate ...ministration, either by increasing the prodrug dose and ...eping the conjugate dose constant at 1 mg/kg/injection ...gure 9B) or by increasing the conjugate dose to 4 mg/ ...injection (Figure 9C). In both cases, the majority of ...mors were cured, again with no evidence of toxicity. ...ally, therapeutic efficacy was also obtained with a 48 ...nterval between conjugate and prodrug administration ...gure 9D). Tumor regressions were obtained in all of ...e mice in these groups, and three of six animals that ...ceived 275 mg/kg/injection CCM were cured. Thus, the ...itumor activities of L49-sFv-bL in combination with ...CM were pronounced, and therapeutic efficacy was ...ieved in a variety of dosing schedules.

## ...SCUSSION

We have previously demonstrated that mAb–bL con...gates activate cephalosporin-containing prodrugs in an ...munologically specific manner and such combinations ...d to regressions and cures of established tumors in ...e (*6, 10, 24, 36*). These conjugates were prepared by ...mbining maleimide-substituted bL with thiol-contain...g mAbs and then subjecting the resulting mixtures to ...rification procedures that involved affinity and size ...clusion chromatographic steps. Although care was ...ken to control the degree of protein modification and ...isolate principally monomeric material, SDS–PAGE ...alysis generally has indicated the presence of ag...gates, dimers and lower molecular mass components ...the conjugate preparations. This is exemplified in ...gure 5D, which shows that chemically produced L49-...'-bL contains several species besides the expected ...oduct at 92 kDa. Such heterogeneity is most likely due ...the lack of specificity inherent in the reagents used ...protein modification (*39*). While a number of elegant ...thods, such as reverse proteolysis (*16, 40–42*) and ...minal amino acid group modification (*43*), have been ...ised to overcome this problem, these techniques can ...lead to considerable product heterogeneity.

An alternative approach toward the preparation of ...form and well-defined antibody–enzyme immunocon...ates has involved recombinant technology for the ...duction of fusion proteins. This has led to the ...elopment of L6-sFv-*Bacillus cereus* β-lactamase (*19*) ...d anti-p185HER2-Fv-*E. coli* β-lactamase (*20*) fusion ...teins, both of which were capable of effecting prodrug ...tivation *in vitro*. More detailed biological studies have ...en reported with a recombinant anti-CEA-Fab-β-glu...ronidase fusion protein, which activated a doxorubicin ...drug *in vitro* and *in vivo* (*4*). The distinguishing ...tures of these fusion proteins are that they are ...mogeneous and potentially can be made in reproduc...le and economical manners. In the work described ...re, we have utilized recombinant methodology for the ...duction of L49-sFv-bL. It was possible to express ...ble fusion protein in *E. coli* such that denaturation ...refolding was not required for activity. L49-sFv-bL ...purified using a two-stage affinity chromatography ...thod leading to the isolation of a homogeneous product ...t was fully active with respect to both the L49 and ...components. As expected, the fusion protein was able ...nd to melanoma cells that expressed the p97 antigen ...d activate a cephalosporin mustard in an immunologi... specific manner.

To minimize systemic, nontargeted drug release *in vivo*, a high mAb–enzyme tumor to normal tissue ratio is needed before the prodrug is administered. To attain the required localization index in mice, the time between conjugate and prodrug administration has varied significantly from one system to another. For example, the delay between conjugate and prodrug was 3 days for 96.5-Fab′-bL (molecular mass 92 kDa) (*10*), 1 week for the anti CEA-Fab-β-glucuronidase fusion protein (molecular mass 250 kDa) (*4*), and 2 weeks for the ICR12–carboxypeptidase G2 conjugate (molecular mass range of 233–316 kDa) (*9*). In some cases, it has even been necessary to accelerate systemic conjugate clearance in a separate step involving the formation of immune complexes before prodrug could be administered (*5, 44, 45*). Here, we have shown that L49-sFv-bL not only clears very rapidly from the systemic circulation (Figure 8A) but also preferentially localizes into subcutaneous tumor xenografts (Figure 8B; Table 2). The very high tumor to nontumor fusion protein ratios obtained within 4–12 h of conjugate treatment would lead to the prediction that, in contrast to other enzyme/prodrug systems (*4, 9, 10*), therapeutic efficacy would not require protracted time intervals between conjugate and prodrug administration. This has now been experimentally confirmed, since cures of established tumors were obtained when CCM was administered 12 h following the conjugate (Figure 9A).

It is noteworthy that a correlation can be made between the outcome in the therapy experiments (Figure 9) and the pharmacokinetic data (Figure 8; Table 2). At a given conjugate dose, the intratumoral concentration decreased with a half-life of approximately 8 h (Table 2). This may be due to a variety of factors such as dissociation of the conjugate from the antigen, membrane recycling, enzyme metabolism, and rapid tumor growth. The net result is that longer time intervals between conjugate and prodrug administration require that the amount of either prodrug or conjugate be increased to maintain therapeutic efficacy (Figure 9).

In conclusion, we have shown that recombinant L49-sFv-bL has properties that are well suited for site-selective anticancer prodrug activation. The fusion protein is homogeneous, localizes in solid tumor masses, and clears very rapidly from the systemic circulation. In these respects, L49-sFv-bL has significant advantages over the L49-Fab′-bL chemically produced conjugate. Finally, we have shown that L49-sFv-bL/CCM combinations lead to cures of established melanoma tumors without toxic side effects. Currently, we are optimizing the treatment protocols and are investigating the effects of L49-sFv-bL/prodrug combinations in several carcinoma tumor models.


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